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Purified mouse autocrine motility factor/phosphoglucose isomerase was found to cause weight loss (cachexia) after 3 days of 3X daily intraperitoneal injection, which was accompanied by significant increases in serum concentrations of the factor. This is a simpler animal model than originally proposed structure of the protein complexed with inhibitor has been soved by x-ray crystallography and published. Mutant forms of the protein have been prepared. Experiments are underway to improve the purity of the recombinant protein and to characterize the effects of the factor on both intact animals and on a mouse muscle cell line in vitro.

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Annual Progress Report, year 01 John M. Chirgwin Ph.D.

Award DAMD17-02-1-0586

Tumor Secreted AMF: Causal Role in an Animal Model of Cachexia

INTRODUCTION:

Update on cancer cachexia, August 2003. Cancer cachexia has three clinical features (Fearon & Moses, 2002; Tisdale, 2002): 1) loss of appetite (anorexia), which probably has a central nervous system component, 2) nutritional mal-absorption, and 3) muscle and fat wasting caused by tumor-stimulated factors (Kotler, 2000; Tisdale, 2000). This application focuses on the 3rd component. A number of factors have been proposed to cause cancer cachexia (Matthys & Billiau, 1997; Tisdale, 1998). These fall into two classes: primary ones produced by the cancer cells themselves and secondary ones, which are inflammatory factors released by the host in response to the tumor. The existence of tumor-produced factors has been long known (Norton et al, 1985), but few such factors have been identified at the molecular level. In addition, cachexia is typical of AIDS, rheumatoid arthritis (Roubenoff et al, 1992), and other diseases, as well as cancer. Despite extensive characterization of cytokine involvement in cachexia, progress in treatment of cancer cachexia has been limited (Argiles et al, 2001; Nelson, 2000), and treatments aimed at inhibiting the actions of host-produced inflammatory mediators have not been widely successful (von Haehling et al, 2002; Inui, 2002). Lack of progress in the area is unfortunate, given the tremendous benefit patients with advanced cancer would receive from effective treatment of cachexia to improve their quality of life and postpone mortality.

Biochemical mechanisms of cachexia have been explored in vivo and in vitro. Lipid wasting (Kalra & Tigas, 2002), changes in the insulin-like growth factor pathways (Crown et al, 2002), and alterations in appetite and caloric balance (Schwartz & Morton, 2002) all contribute to cancer cachexia. However, muscle wasting is the facet which is currently best understood (Tisdale, 2001), as well as being amenable to scientific study and, potentially, to therapeutic intervention. The severe skeletal muscle wasting characteristic of cancer cachexia appears to be due to activation of proteasomal degradation of structural proteins in muscle (Hasslegren & Fisher, 2001; Whitehouse et al, 2001: Giordano et al, 2003). Less understood are the primary factors released by tumor cells responsible for initiating the muscle wasting, but progress in the last several years has identified several candidates: proteolysis-inducing factor (PIF), a sulfated polypeptide isolated from urine of cachectic patients (Cabal-Manzano et al, 2001; Lorite et al, 2001), and myostatin (Zimmers et al, 2002).

In addition, the osteolytic factor PTHrP causes cachexia when systemically elevated. However, this is accompanied by humoral hypercalcemia of malignancy (Guise et al, 1992; Guise et al, 1996; Guise & Mundy, 1998), and there may be direct cachectic effects of PTHrP on the kidney, independent of hypercalcemia (Iguchi et al, 2001). Complicating the role of PTHrP is its contribution to osteolytic bone metastases by breast cancer. The MDA-MB-231 cell line causes PTHrP-dependent bone metastases (Guise et al, 1996). Animals with bone metastases due to this tumor become profoundly cachectic but without significant increases in circulating concentrations of PTHrP (Yin et al,1999). Recent work also implicates cachectic effects on

mitochondria by activation of uncoupling proteins (UCPs), especially UCP-3, which results in ATP energy wasting (Tisdale, 2002; Glass, 2003).

Role of the proteasome. Skeletal muscle proteolysis in cachexia is probably due to increased activity through the proteasomal pathway, rather than via lysosomes or soluble sarcoplasmic proteases Lazarus et al, 1999). It has recently been observed that certain cancer treatment protocols can either enhance (Tohgo et al, 2002) or inhibit this muscle degradation (Tilignac et al, 2002) pathway. Thus, cancer chemotherapy may alter cachexia in patients. Omega-3 fatty acids and other eicosanoids can regulate the activity of the proteasome (Whitehouse et al, 2001), providing a biochemical rationale for the dietary treatment of cancer cachexia (Ross & Fearon, 2002; Jho et al, 2002). It is not yet clear that activation of proteasomal degradation is the central or the only pathway for muscle wasting in cancer cachexia (Hasslegren et al, 2002; Jagoe & Goldberg, 2001; Glass, 2003; Lecker, 2003). In addition to their effects on the proteasome, ω-3 fatty acids decrease expression of ras, AP-1, and cycloxygenase-2 (Hardman, 2002).

BODY OF PROGRESS REPORT

Timetable: The award of this grant was made just as the Principal Investigator was moving from the University of Texas to the University of Virginia. Initial work was commenced upon arrival in Charlottesville Virginia in October 2002. A new research associate was recruited to work on this project, Ms. Lisa Wessner, who is an experienced molecular biologist. She has learned all of the techniques specific to the project, which has been fully active since approximately January 1 of the current year. Thus, this progress report represents work carried out over a seven-month period. All animal procedures are now in place and approved by the insitution (an extremely slow process).

The revised application contained **3 Specific Aims and 9 Tasks** in the revised Approved Statement of Work.

Under Aim1, **Task 1** is complete and **Task 2** is underway. Our initial data **(Table 1)** showed that Alzet minipumps did not achieve useful increases in steady-state blood concentrations of mouse PGI/AMF, even when the pumps were loaded with 10mg/ml protein solution. However, initial experiments **(Figure 1)**, demonstrate that a simpler approach successfully gave substantially increased steady-state blood concentrations of mouse PGI/AMF. In this experimental protocol, animals were given the factor as sterile intraperitoneal (i.p.) injections of protein in PBS at 8 AM, noon, and 4 PM. Blood levels of PGI/AMF were measured at the 4PM time. **Figure 1** indicates that the injected AMF/PGI was entirely cleared from the blood stream by 24 hours. In this experiment, there was a decrease in body mass consistent with a significant cachectic effect of the injected factor. This response is equivalent to that seen by the Tisdale group in their experiments with PIF, a sulfated peptide purified from urine of cachectic animals (Todorov et al, 1997; Lorite et al, 1998). When injected into animals the peptide reproduced cachexia. No cDNA sequence for PIF has been published, but a commercial patent (Akerblom & Murry, 1998) describes a cDNA, which includes the reported N-terminal sequence of PIF (Todorov et al, 1997). This sequence does not give any significant matches in the present Genbank database of human and mouse sequences when subjected to a BLAST search (Chirgwin, unpublished), suggesting that PIF/HCAP may be produced by an opportunistic

microorganism. Chlamydial infection, for example, may contribute to cachexia in patients with AIDS, and in general patients with cachexia are immunocompromised. Recent works shows that PIF can activate, through NF-6B, endothelial cell expression of IL-6 and IL-8 (Watchorn et al, 2002), the later of which is produced by breast cancer cells and can directly enhance bone metastases (Bendre et al, 2002).

On the basis of the initial results, shown in **Figure 1**, the remaining experiments for Tasks 2, 4, 7 and 8 will be carried out by direct i.p. injection of recombinant protein 3X per day. An initial experiment to study clearance of a single injection of purified protein (**Figure 2**) suggests that between 10 and 100ug/ injection should be sufficient, which would be at least 5X less than the amount used in Figure 1. We are presently determining the minimum effective dose to give progressive weight loss accompanied by increased steady-state blood concentrations. As soon as this is determined, we will test whether the number of doses (presently 9 = 3/day x 3 days) can be decreased. This modified approach eliminates the need for animal surgery to implant minipumps and permits the experiments to be of one week or less duration. The number of animals remains unchanged. The results also indicate that expensive Balb/c nude/nude mice are not needed for the cachectic response.

Tasks 3 and 4 have not been initiated.

Tasks 5 and 6 have been started. The catalytically inactive mutant E357A has been constructed, expressed and purified. The role of isomerase (PGI) activity in relation to autocrine motility factor (AMF) activity remains a central controversy in the field, with two papers reporting AMF activity as a property of bacterial PGI (Sun et al, 1999; Chou et al, 2000). In addition PGI catalytic activity has been suggested to be essential to AMF cytokine activity. These experiments involved adding PGI active site inhibitors at mM concentrations into bioassays, in which the AMF/PGI factor was added at nM concentration. The million-fold excess of inhibitor over factor could easily have resulted in non-specific inhibitory effects (e.g., Lagana et al, 2000). In fact, more recent experiments have suggested the opposite (Tsutsumi et al, 2003). Much of the AMF cytokine work has not taken into account the current knowledge of PGI structure. We (Davies et al, 2003) and others (Arsenieva & Jefferey, 2002), have shown that ligand binding to mammalian PGIs results in only very small conformational changes in the surface of the protein away from the active site (where binding to the AMF receptor almost certainly takes place).

In **Task 6**, we have encountered a substantial obstacle. The recombinant proteins upon which all of the experiments in the proposal rely are expressed in the bacterium *Escherichia coli*. Gram negative bacteria are a prime source of inflammatory endotoxins collectively called lipopolysaccharides (LPS). We have assayed all of our AMF/PGI preparations with an endotoxin assay kit using amoebocyte lysates from Sigma Chemical Co (St. Louis). By this assay all of our preparations were LPS-free. However, the standard curves with the Sigma kit gave inconsistent results, and we have switched to a parallel assay from BioWhittaker, CA). By this assay, our preparations (such as that used in the supplied preliminary data) were not LPS-free, although the level of contamination was that considered by other investigators to be relativelylow (Bausinger et al, 2002). LPS contamination has been realized to cause cytokine-like artifactual responses in mammalian cells treated with bacterially expressed proteins (Gao & Tsan, 2003; Bausinger et al, 2002; Colangeli et al, 1998; Ozaki et al, 1989).

Preparation of LPS-free AMF/PGI. We have tested several different types of metal chelates resins for purification of His6-tagged protein. Standard NiNTA agarose gives material that appears substantially pure by Laemmli gel with Coomassie blue staining (**Figure 3**). The

columns yielded AMF/PGI preparations with equivalent amounts of LPS contamination. Addition of washing steps with nondenaturing detergents, such as sodium deoxycholate or triton X-100 was also ineffective. A published procedure for this purpose, involving washing the column with cold isopropanol (Kees et al, 2000) was totally unsatisfactory. The isopropanol interferes with the column flow and was entirely without effect on reduction of the endotoxin contamination of the eluted protein.We have been successful in removing LPS from AMF/PGI preparations by adding a second chromatography step of passing the purified protein in PBS over a column of immobilized polymyxin B (Detoxigel, Pierce Chemical Co). Polymyxin B is a cyclic oligopeptide antibiotic effective against gram-negative bacteria; it binds bacterial lippolysaccharides with high affinity. The Detoxigel step results in loss of almost all of the applied AMF/PGI and we have been able to purify only about 1mg of protein in this manner. Prior to Detoxigel chromatography the contamination of AMF-PGI was 1.34 parts per million (ppm) on a weight per weight basis, using the conversion factor of 1 I.U. of endotoxin = 83 pg (Kees et al, 2000). After chromatography , the contamination was 0.079 ppm, representing a 60-fold purification. The material prior to Detoxigel purification contains 90 I.U./mg of AMF-PGI, while 60 I.U./mg is defined as low endotoxin contamination of r(hu)hsp 70, which lacks activity on monocytes in vitro (Bausinger et al, 2002).

We have tested our most highly purified AMF-PGI in chemotaxis assays with two mouse monocyte/macrophage cell lines. The data shown in **Figure 4** , show that the material was entirely negative in these two assays. These experiments were conducted in collaboration with Prof. Lynda Bonewald, University of Missouri Kansas City School of Dentistry.

We are currently testing two further strategies: 1) initial binding to and washing of AMF/PGI to the Ni-NTA agarose affinity chromatography resin in the presence of soluble polymyxin B to dissociate the contaminating LPS from the resin-bound AMF/PGI; 2) active-site affinity chromatography as originally described by Phillips et al (1976). The active site of the protein binds to washed phosphocellulose and specific elution is accomplished with glucose 6-phosphate substrate. We will also test whether combining 1) and 2) is effective. We believe that this is an important problem to solve. Unrealized LPS contamination has resulted in major published artifacts with other proteins. We suspect that the AMF activity reported for bacterial PGI (Sun et al, 1999; Chou et al, 2000) is probably due to LPS contamination, a possibility supported by recent, more careful work (Amraei & Nabi, 2002), which has invalidated the earlier conclusions.

We believe that the additional work proposed within this task could have general applicability for the field of biological activity of bacterially-expressed proteins. If the new purification steps are not successful, we will use the inefficient approach of Detoxigel chromatographt or of injection of less pure AMF/PGI which has been mixed with sterile USP-grade polymixin B (Sigma).

An in vitro model of muscle wasting was recently described by Gomes-Marcondes et al (2002) have described, in which PIF directly stimulates the hydrolysis of radiolabeled muscle protein from the myoblast/myotube cell line C2C12 in vitro. This model provides an efficient system for biochemical assay of circulating factors which act directly on muscle cells. The C2C12 cell line progresses through a skeletal muscle differentiation program in vitro. A mediator of this process is MyoD, which is in turn regulated by the transcription factor NF-6B. The cachectic cytokines TNF" and IFN-(may cause muscle wasting by suppressing MyoD expression (Guttridge et al, 2000) in C2C12 cells. PIF can regulates transcription via NF-6B and

STAT2 (Watchorn et al, 2001) while the activity of NF-6B is regulated by the proteasome (Langen et al, 2001). The factor also plays a central role in multiple myeloma (Berenson et al, 2001; Hideshima et al, 2002). Suppression of NF-6B attenuates cachexia and metastasis in several mouse tumor models (Arlt & Schafer, 2002). Thus the NF-6B transcription factor may also be a target for anti-cachexia treatments, while itself being one of the mediators of the actions of proteasome inhibitors (Mitch & Price, 2000; Adams, 2001; Tisdale, 2002a).

We have attempted to replicate the Gomes-Marcondes model, although using IL-6 as an inducer of cellular proteolysis, since PIF is unavailable. We conclude that the model is probably acceptable as a means of analyzing responses in vitro to factors which stimulate muscle wasting in vivo. However, the model is technically unsatisfactory. Inspection of the original paper reveals large statistical errors, with large n values of 8 or greater needed to achieve statistical significance with small changes in total protein. We believe the model can be substantially improved by analyzing protein wasting by a more traditional analysis using trichloracetic acid precipitation to distinguish high molecular weight labeled protein from the soluble oligopeptides released by stimulated proteolysis. Similar approaches have been applied by other to C2C12 protein degradation (Taylor et al, 2001; Thompson et al, 1996; Fernandez & Sainz, 1997), although not in the context of assaying cachectic factors.

This is a supplemental experiment within task 2. If successful, the methodology would permit analysis of muscle-targeting cachectic factors in vitro, decreasing the future need for animal experiments.

KEY RESEARCH ACCOMPLISHMENTS:

1) Animal model of direct i.p. injection of AMF/PGI established.
2) Preliminary validation of central hypothesis obtained: Injected AMF/PGI caused progressive weight loss of 10% over the course of 4 days in individual mice.
3) Mutant mouse AMF/PGI constructed, expressed, and purified
4) Unsuspected contamination of AMF/PGI with inflammatory bacterial endotoxin detected. Improved purification protocol under development.
5) Crystal structure of mammalian AMF/PGI with active-site-bound ligand solved and published.

REPORTABLE OUTCOMES:

One manuscript published:

Davies C, Muirhead H, Chirgwin J (2003). The structure of human phosphoglucose isomerase complexed with a transition-state analogue. Acta Crystallogr D Biol Crystallogr 59:1111-1113

Four manuscripts in press accepted for publication which include reviews of the contributions of bone metastases to cancer cachexia:.

Chirgwin JM, Guise TA. Role of TGFb in osteolytic bone metastases. Clin Orthop, in press, 2003.

Chirgwin JM, Guise TA. Bisphosphonates in prostate cancer bone metastases. Semin Oncol, in press, 2003.

Chirgwin JM, Guise TA. Molecular mechanisms of cancer metastases to bone. Curr Opin Orthop, in press, 2003.

Guise TA, Chirgwin JM. Biology of bone metastases. Chapter in Diseases of the Breast, 3rd edition. Harris, Lippman, Morrow, and Osborne (eds). Lippincott Williams & Wilkins, accepted for publication, 2003.

CONCLUSIONS

Purified mouse autocrine motility factor/phosphoglucose isomerase was found to cause weight loss (cachexia) after 3 days of 3X daily intraperitoneal injection, which was accompanied by significant increases in serum concentrations of the factor. This is a simpler animal model than originally proposed. Thus the main hypothesis of the original proposal appears to be correct. Progress in the first (partial) year is on track, despite relocation of the laboratory from University of Texas to University of Virginia. Statistical validation of the initial animal model observations will be carried out in year 02

Structure of the protein complexed with inhibitor has been solved by x-ray crystallography and published. Mutant forms of the protein have been prepared. Experiments are underway to improve the purity of the recombinant protein and to characterize the effects of the factor on both intact animals and on a mouse muscle cell line in vitro.

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LEGENDS TO TABLE AND FIGURES

Table 1. Animals were implanted under anesthesia with osmotic minpumps as described in the original proposal. Pumps were loaded with 1 or 10 mg/ml sterile AMF/PGI stock in PBS. At 4 PM each day AMF/PGI was assayed on 10 ul of serum obtained from a retro-orbital blood sample obtained under anesthesia. Numbers immediately below the animal weights in g in each box are the raw PGI catalytic rate values. The results indicate that the minipumps failed to give significant increases in the serum concentrations of AMF/PGI, compared to those seen in patients with bone metastases or cachexia (Bodansky, 1954).

Figure 1. In the experimental protocol, animals were given the factor as sterile intraperitoneal (i.p.) injections of protein in PBS at 8 AM, noon, and 4 PM. Blood levels of PGI/AMF were measured at the 4 PM time. Animals were weighed at the indicated times. Animals were injected on days 1, 2, and 3.

Figure 2. Protocol was similar to that described under Figure 1. Mice received a single bolus i.p. injection of mouse AMF/PGI. 50 ul aliquots of blood were obtained retro-orbitally under anesthesia at the indicated times and assayed for PGI activity in 10 ul of serum.

Figure 3. Equivalent aliquots of column fractions of effluent from a Qiagen NiNTA column loaded with the cleared supernatant of E coli BL21DE3 pLysS cells treated with iPTG to induce expression of mouse PGI-H6 as described in the original proposal. 12.5% denaturing SDS Laemmli gel stained with Coomassie blue R250 and photographed with Kodak EDAS digital gel documentation syytem. Samples boiled with 2-mercaptoethanol reducing agent. Major band is the correct size for the anticipated subunit of 66 kDa.

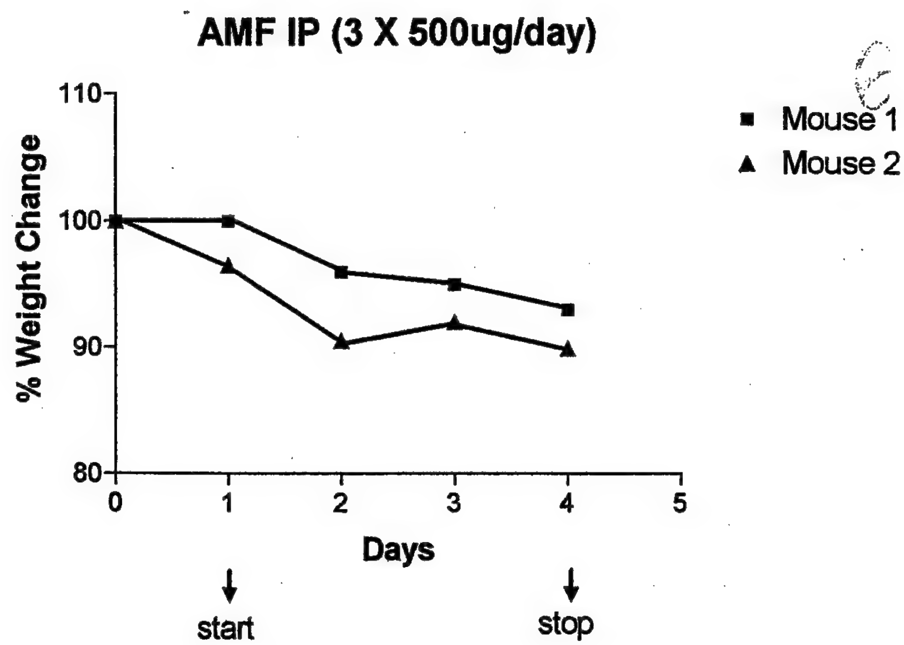
Figure 4. Chemotaxis assays. RAW 264.7 and MOPC-5 are standard mouse monocyte/macrophage cell lines from the ATCC. Positive control, last lane of each panel, is media conditioned by the mouse osteocyte cell line MLO-Y4, developed by the collaborator in these experiments, Dr. Lynda Bonewald, University of Missouri Kansas City School of Dentistry.

8/13/02 Mouse AMF Pump Experiment

Pumps: Hold ~ 0.2 mL, delivery ~ 1uL/hour

	Mouse 1 PBS NC	Mouse 2 1mg/ml VC	Mouse 3 1mg/ml BC	Mouse 4 10mg/ml LC	Mouse 5 10mg/ml RC
Day 0	34.95g	31.94g	40.25g	34.60g	32.50g
Day 1	33.60g 0.1884Δ/min	31.80g 0.1207Δ/min	39.30g 0.0719Δ/min	35.55g 0.2799Δ/min	31.45g 0.2304Δ/min
Day 2	33.74g 0.1341Δ/min	32.33g hemolysed	40.00g hemolysed	34.60g 0.2346Δ/min	31.81g hemolysed
Day 3	33.13g 0.1983Δ/min	32.50g 0.1712Δ/min	39.63g 0.0759Δ/min	34.30g 0.1486Δ/min	32.30g 0.1426Δ/min
Day 4	32.98g 0.1335Δ/min	33.12g 0.1136Δ/min	38.76g 0.0712Δ/min	35.30g 0.2142Δ/min	32.65gg 0.2345Δ/min
Day 7	33.80g 0.1129Δ/min	33.27g 0.1256Δ/min	39.75g 0.0750Δ/min	33.70g 0.2244Δ/min	32.40g 0.2822Δ/min

Table 1



Serum AMF Activity

	Mouse 1	Mouse 2
Baseline	17U/ml	11U/ml
Day 1	1564U/ml	1800U/ml
Day 2	2900U/ml	1550U/ml
Day 3	1488U/ml	1690U/ml
Day 4	4U/ml	10U/ml

Figure 1

Clearance of I.P. PGI

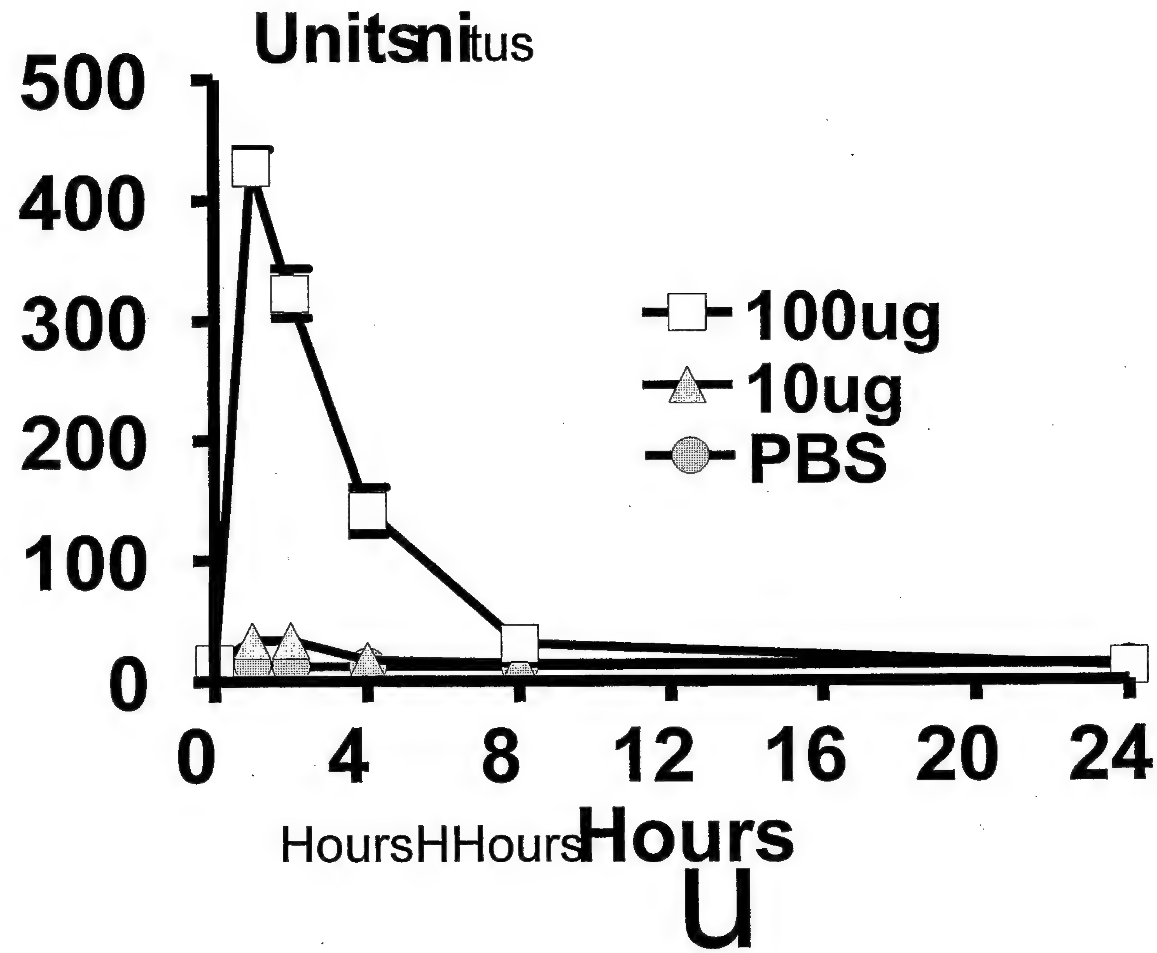
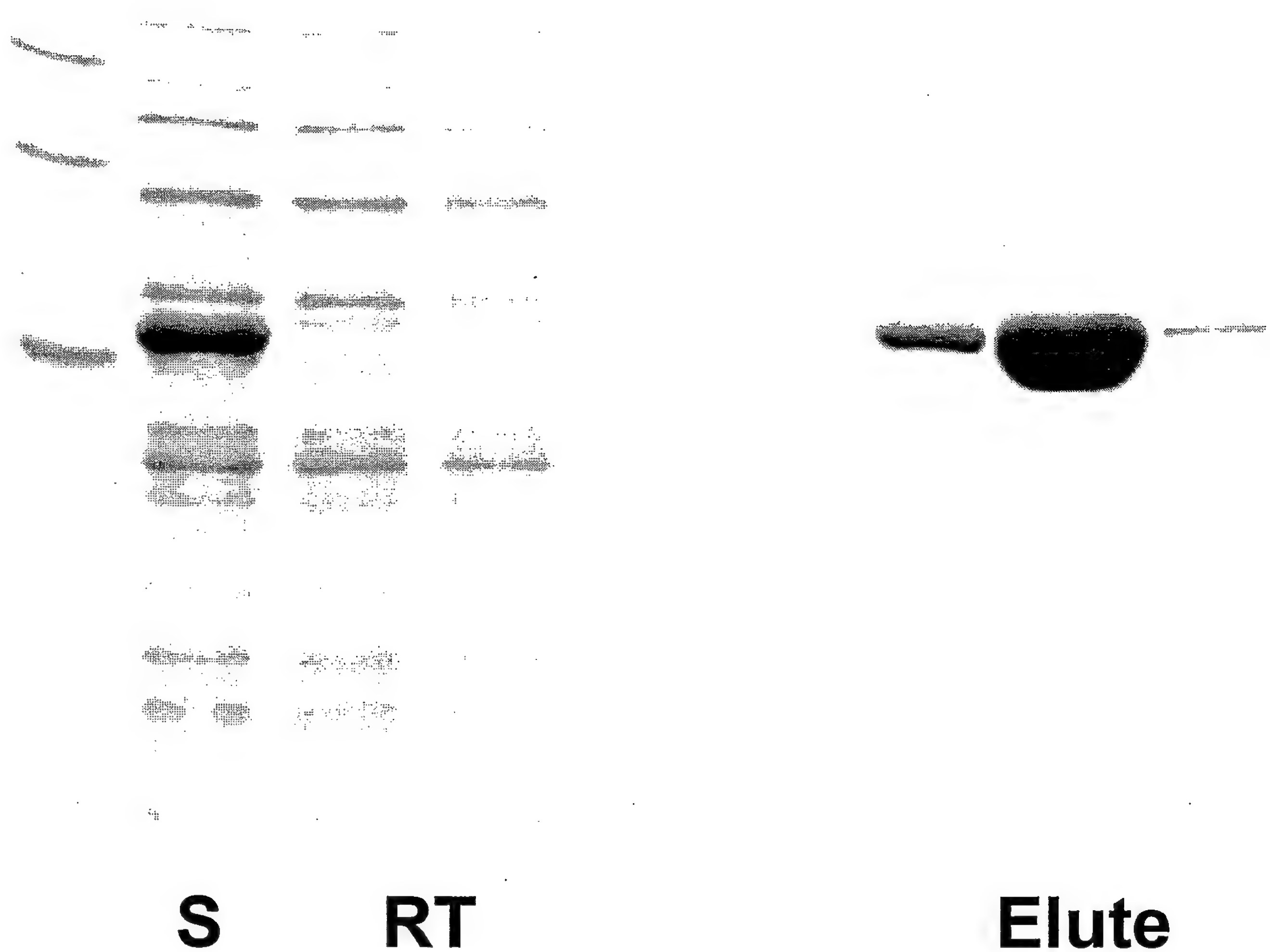


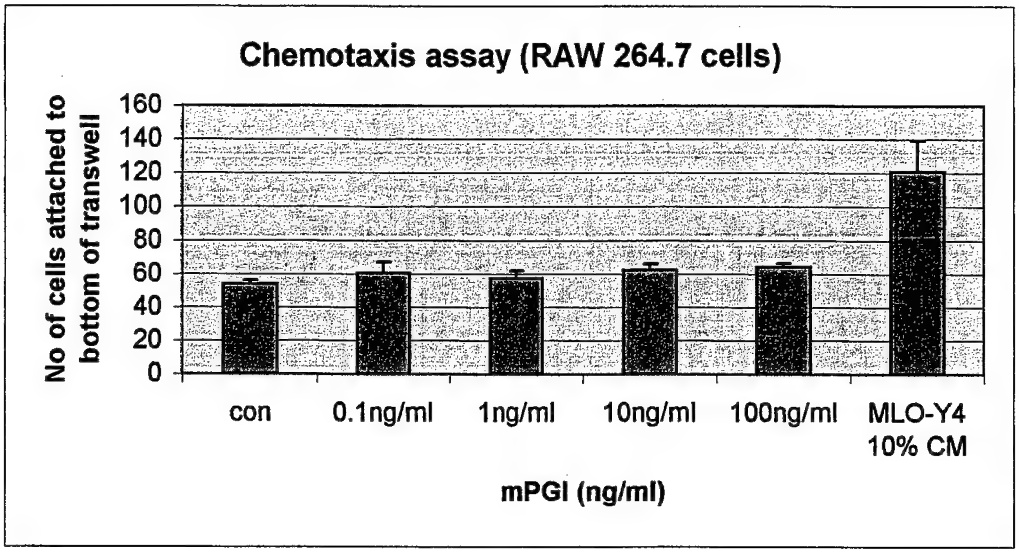
Figure 2

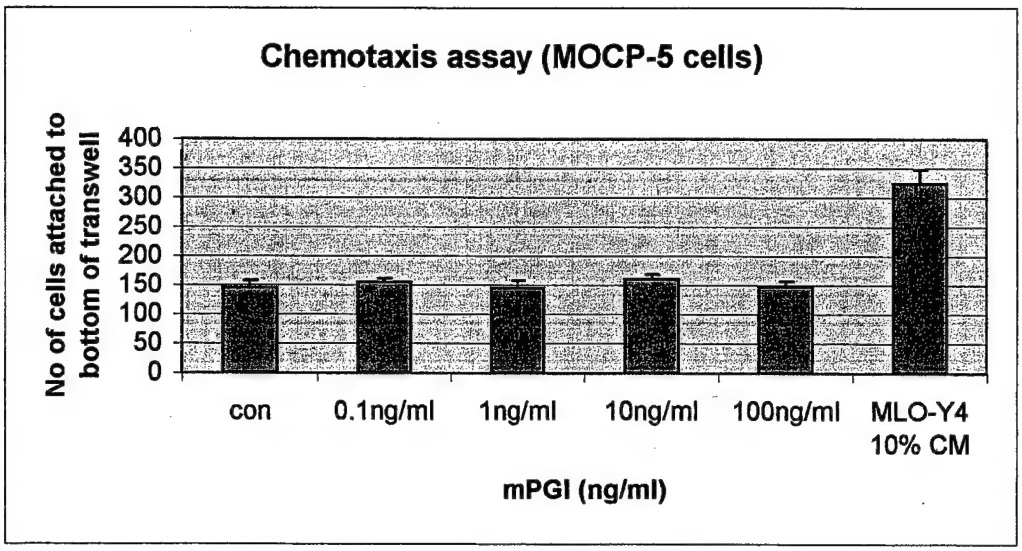
Mouse PGI-H_6 Purification



p 21

Figure 3


Chemotaxis assay (RAW 264.7 cells)
No of cells attached to bottom of transwell
160
140
120
100
80
60
40
20
0
con
0.1ng/ml
1ng/ml
10ng/ml
100ng/ml
MLO-Y4 10% CM
mPGI (ng/ml)


Chemotaxis assay (MOCP-5 cells)
No of cells attached to bottom of transwell
400
350
300
250
200
150
100
50
0
con
0.1ng/ml
1ng/ml
10ng/ml
100ng/ml
MLO-Y4 10% CM
mPGI (ng/ml)

APPENDIX

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The structure of human phosphoglucose isomerase complexed with a transition-state analogue

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Phosphoglucose isomerase (PGI) is a workhorse enzyme of carbohydrate metabolism that interconverts glucose 6-phosphate and fructose 6-phosphate. Outside the cell, however, the protein appears to function as a cytokine. A crystal structure of human PGI bound with 5-phosphoarabinonate, a strong inhibitor that mimics the *cis*-enediol(ate) intermediate of the reaction, has been determined at 2.5 Å resolution. The structure helps to confirm the assignment of Glu357 as the base catalyst in the isomerase reaction.

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PDB Reference: phosphoglucose isomerase–5-phosphoarabinonate, 1nuh, r1nuhsf.

1. Introduction

Phosphoglucose isomerase (EC 5.3.1.9) is a workhorse enzyme of sugar metabolism. It catalyses the second step of glycolysis, the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P), by transfer of a proton between the C2 position of G6P and C1 of F6P (Rose, 1975). Recent crystal structures of the enzyme have led to proposals that Glu357 is the active-site base responsible for this transfer (Lee *et al.*, 2001; Read *et al.*, 2001) and that either His388 or Lys518 catalyses the ring-opening of the sugar substrate (Davies & Muirhead, 2003; Lee *et al.*, 2001). The human enzyme is of medical interest because mutations in this enzyme lead to non-spherocytic haemolytic anaemia (Baughan *et al.*, 1968) and because high levels of PGI activity are measured in the sera of patients with certain cancers (Baumann *et al.*, 1990).

Interest in PGI has grown following the discoveries that it manifests cytokine function in a wide variety of cellular activities (Gurney *et al.*, 1986; Watanabe *et al.*, 1996; Xu *et al.*, 1996) and appears to be an antigen in rheumatoid arthritis (Matsumoto *et al.*, 1999) and sperm agglutination (Yakirevich & Naot, 2000). To what extent the enzymatic properties of PGI overlap with its cytokine functions remains unclear.

Here, we present the crystal structure of human PGI bound to a transition-state analogue, 5-phosphoarabinonate (PAB), solved at 2.5 Å resolution. Along with equivalent structures obtained from pig and rabbit sources (Davies & Muirhead, 2002; Jeffery *et al.*, 2001), it supports the hypothesis that Glu357 is the base catalyst in the reaction mechanism.

2. Experimental

Human PGI was purified and crystallized as described previously (Read *et al.*, 2001) except that 5 m*M* PAB was included in the protein drops. The resulting crystals were of the same morphology as native crystals but diffracted X-rays less well. After stabilization in a solution containing 2.1 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.5 and 30% glycerol, the crystals were flash-frozen to 100 K. Data were collected with an R-AXIS IV^{++} detector positioned at a crystal-to-detector distance of 160 mm and mounted on an RU3-HBR X-ray generator (Rigaku-MSC) fitted with Osmic mirror optics. The crystals belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 94.4$, $c = 137.1$ Å. A total of 173 frames were collected in 0.5° oscillations to ensure high redundancy, with an exposure time of 5 min per frame. The data were processed using *d*TREK* (Pflugrath, 1999). The starting model for refinement was the 1.6 Å resolution structure of human PGI (Read *et al.*, 2001) from which a bound sulfate and all waters molecules had been removed. After initial refinement using *X-PLOR* (Brünger, 1992), both $2(|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ electron-density maps clearly showed the PAB molecule bound at the active site. After a molecule PAB was fitted to the density, subsequent rounds of refinement used *REFMAC* (Murshudov *et al.*, 1997). The final model is numbered 1–555 and includes one PAB molecule, six sulfate mole-

Table 1
X-ray diffraction data and refinement statistics.

Values in parentheses are for the outer resolution shell.

Data collection	
Resolution range (Å)	50–2.5 (2.59–2.5)
R_{merge}† (%)	12.8 (25.4)
Redundancy	6.9 (6.8)
Completeness (%)	98.4 (99.8)
$\langle I \rangle / \langle \sigma(I) \rangle$	5.7 (3.1)
Refinement	
Resolution range (Å)	50.0–2.5
σ cutoff applied	0.0
Total No. of reflections	21468
Reflections used in R_{free} (%)	5.0
No. of non-H protein atoms	4424
No. of sulfate molecules	6
No. of water molecules	109
R factor (%)	21.7
R_{work} (%)	21.4
R_{free} (%)	26.8
R.m.s. deviations from ideal stereochemistry	
Bond lengths (Å)	0.011
Bond angles (°)	1.48
B factors (Å^2)	
Overall B factor	25.16
Mean B factor (main chain)	24.55
R.m.s. deviation in main-chain B factor	0.390
Mean B factor (side chains and waters)	25.72
R.m.s. deviation in side-chain B factors	1.454
Ramachandran plot statistics (%)	
Residues in most favoured region	88.8
Residues in additionally allowed regions	10.8
Residues in generously allowed regions	0.4
Residues in disallowed regions	0.0

† $R_{merge} = \sum |I_i - I_m| / \sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry-related reflections.

cules (arising from the crystallization solution) and 109 water molecules. The data-collection and refinement statistics are shown in Table 1.

3. Results and discussion

3.1. Structure description

PGI has been solved from a variety of mammalian sources and from *Bacillus stearothermophilus* in both native and inhibitor-bound forms (see, for example, Davies & Muirhead, 2002; Jeffery *et al.*, 2000; Read *et al.*, 2001; Sun *et al.*, 1999). The protein architecture is essentially identical in mammalian PGIs and is highly similar in the enzyme from *B. stearothermophilus*. The structure comprises two domains, termed large and small, where each domain consists of a central β-sheet surrounded by α-helices. The active site is located in a crevice between the large and small domains, near the subunit boundary. The enzyme form of human PGI exists as a dimer (Tilley *et al.*, 1974), but since it crystallizes as a monomer in the asymmetric unit a symmetry operation is required to generate the dimer. The active site comprises residues that are likely to play a role in the catalytic mechanism, including Glu357, Arg272, His388 and Lys518. One of these residues, His388, is contributed by the adjacent monomer.

3.2. Ligand binding

PAB is a competitive inhibitor of PGI that is believed to mimic the *cis*-enediolate intermediate of the catalytic reaction (Chirgwin & Noltmann, 1975). Our structure of human PGI in complex with PAB helps to further resolve the ambiguity regarding the binding mode of this inhibitor. The PAB molecule is bound to the active site in essentially an identical manner to that seen in equivalent complexes of PGI from rabbit (Jeffery *et al.*, 2001) and pig (Davies & Muirhead, 2002), but the opposite of that seen in a complex with PGI from *B. stearothermophilus* (Chou *et al.*, 2000) (Fig. 1). As expected, the sulfate molecule that was observed in the active site of the native structure (Read *et al.*, 2001) has been displaced by the phosphate group of the PAB inhibitor. The phosphate group is oriented by the same cluster of serine and threonine side chains (Ser209, Thr211, Thr214 and Ser159) as well as the amide N atoms of Lys210 and Thr211 and by one water molecule to Thr217. Both the C2 and C3 hydroxyls (equivalent to C3 and C4 of the substrate) are within hydrogen-bonding distance of the amide group of Gly158. This region of the inhibitor lies close to the turn formed by Gly157 and Gly158 and the absence of side chains in these positions facilitates a closer binding of the substrate. The monitoring of these two hydroxyls by Gly158 probably contributes to the high specificity of PGI for its sugar substrates. One of the side-chain O atoms of Glu357 lies close to O1A and C1 of PAB as well as to the guanidinium group of Arg272. This arrangement suggests that Glu357 is best placed to abstract a proton from the C2 and C1 positions of G6P and F6P, respectively, as proposed recently (Lee *et al.*, 2001; Read *et al.*, 2001), and that the positive charge of Arg272 may stabilize the negative charge of the *cis*-enediolate intermediate. Lys518 and His388 both contact O4, which is equivalent to the ring oxygen of the substrate, and Lys518 also contacts O5. Either or both of these residues may participate in ring opening.

3.3. Comparison with native human PGI

Two structures of human PGI have been published. The first of these contains a sulfate in the active site that appears to mimic the phosphate moiety of the substrate (Read *et al.*, 2001), whereas the second structure is free of ligands and so better represents the true native state of the enzyme (Tanaka *et al.*, 2002). Comparisons of the sulfate-bound structure with a ligand-free structure of rabbit PGI suggested that elements of the small domain shift from an 'open' to 'closed' conformation upon binding sulfate (Read *et al.*, 2001). The hypothesis that the sulfate moiety was mimicking the sugar phosphate is confirmed by the human PAB-bound structure, in which the same region of the small domain is seen in the 'closed' conformation. In contrast, all four molecules present in the

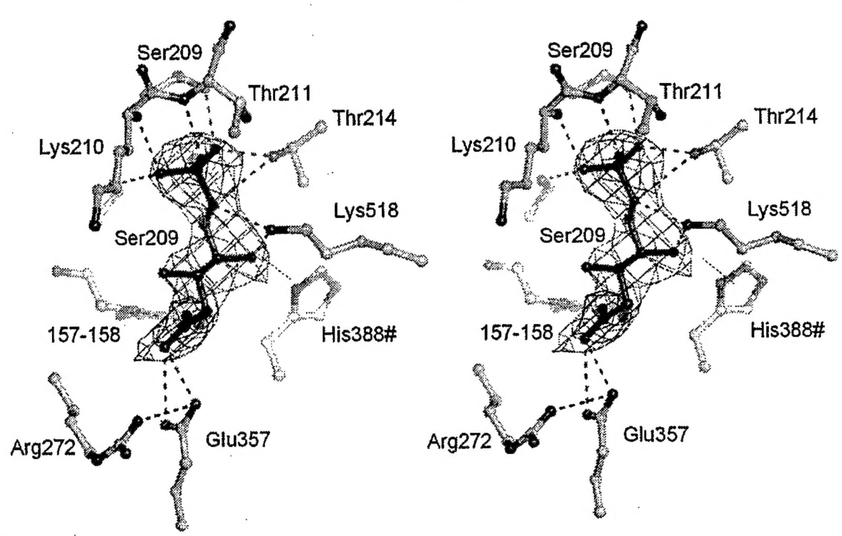

Figure 1
5-Phosphoarabinonate bound to human phosphoglucose isomerase at 2.5 Å resolution. A stereo picture of the active-site region, showing the $2(|F_o| - |F_c|)$ electron density of the bound inhibitor, contoured in blue at 1σ. The active-site residues and inhibitor molecules are shown in ball-and-stick form. The inhibitor is coloured red. This figure was prepared using *PyMOL* (DeLano, 2002).

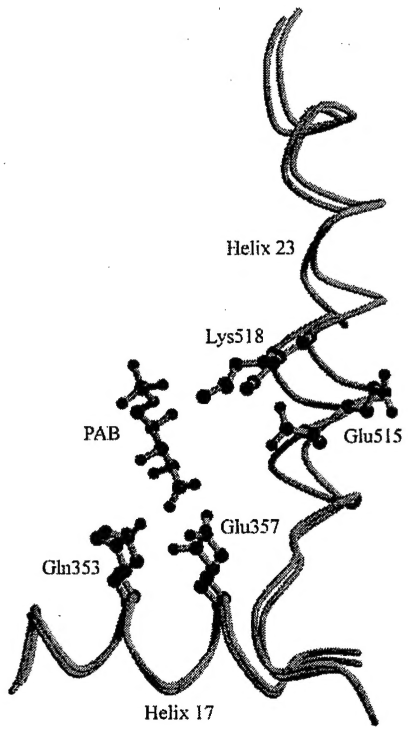

Figure 2
The shift of α23 caused by the binding of 5-phosphoarabinonate. Shown is a backbone superimposition of the sulfate-bound human structure (Read *et al.*, 2001) coloured in orange and the PAB-bound structure in green. Important residues are shown in ball-and-stick representation, in which the bonds are coloured the same as the backbone. Note the rotation of the carboxylate of Glu357 and the slight shift of the adjacent residue, Gln353. For clarity, only helices 23 and 17 are shown. This figure was prepared using *MOLSCRIPT* (Kraulis, 1991).

structure of a ligand-free human PGI are seen in the 'open' conformation (Tanaka *et al.*, 2002).

The only other structural difference of significance is the shift of the N-terminal half of helix α23 (residues 512–520) toward the active site which occurs in the PAB-bound structure but in neither of the human native structures (Fig. 2). The same movement of α23 has been seen in the pig and rabbit homologues of PGI (Arsenieva & Jeffery, 2002; Davies & Muirhead, 2002, 2003). The shift of Lys518 toward the active site and its close proximity to the ring oxygen is concordant with a role for this residue in ring opening. Interestingly, the carboxylate group of Glu357 rotates by approximately 90° to align more closely with the carboxylate on the PAB molecule. In the case of the true substrate, a similar repositioning would enhance the ability of Glu357 to abstract a proton from the C1/C2 positions (Fig. 2).

4. Conclusion

The structure of human PGI bound to 5-phosphoarabinonate further establishes Glu357 as the best candidate for base catalyst, as proposed recently (Lee *et al.*, 2001; Read *et al.*, 2001), supplanting earlier suggestions that His388 was responsible (Chou *et al.*, 2000; Jeffery *et al.*, 2000). Instead, His388 is likely to be the acid catalyst for ring opening. The close proximity of Lys518 to the ring oxygen and its shift towards the active site upon PAB binding suggest that it too has a role in the mechanism of ring opening. PGI is becoming increasingly better characterized as an enzyme, but much remains to be elucidated regarding its cytokine function.

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